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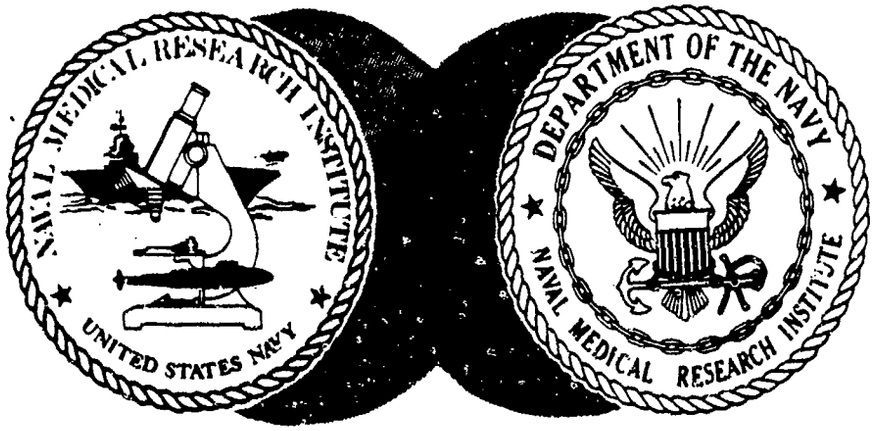

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POLYCLONAL ACTIVATION OF MURINE LYMPHOCYTES BY ANTIBODY TO CELL SURFACE IGD

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The availability of hybridoma produced anti-δ antibodies and murine IgD secreting plasmacytomas made it possible to examine in vivo activation of murine lymphocytes by anti-δ antibodies using anti-δ concentrations similar to those achieved in in vitro activation. Within 24 hrs after i.v. injection of 800 µg of affinity purified goat anti-mouse δ (GaMδ) antibody splenic B cells increased their quantities of cell surface (s) Ia antigen, their size, and their rate of DNA synthesis. All three processes are T-independent.

The data suggest that in vivo B cell activation by scluble anti-δ proceeds through two phases. The first, includes increases in sIa density, cell size, and rate of DNA synthesis and appears to be T-independent. The second phase, includes a further increase in the rate of DNA synthesis, acquisition of sIgG, and Ig secretion, appears to be T-dependent and, to a large extent, carrier dependent, which suggests that T cell help specific for goat Ig may be polyclonally focused onto B cells by GaMδ antibody.

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POLYCLONAL ACTIVATION OF MURINE LYMPHOCYTES BY ANTIBODY TO CELL SURFACE IgD

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INTRODUCTION

The discovery that IgD is present on most human B lymphocytes[1-3] was rapidly followed by its identification as a major B lymphocyte surface isotype in several other mammalian species,[4-7] as well as by attempts to ascertain its function. Many of these efforts have used antibodies prepared against IgD as functional probes with the thought that anti-δ would reproduce polyclonally the changes induced in B cells when their surface IgD bound antigen. In vitro experiments have shown anti-δ antibodies to block antigen specific responses to a variable extent[8-10] and to induce B lymphocytes to proliferate.[11-14] Such cells, when further stimulated by macrophage or T cell derived factors, differentiate into antibody secreting cells.[14] While the culture conditions and physical forms of anti-δ antibodies required to achieve B cell activation have varied, it is generally agreed that high anti-δ concentrations (i.e., 50-100 μg/ml) are required to maximize B cell proliferation.[11-13]

In vivo experiments performed in monkeys,[15-17] rats,[18] and mice[10,19] have generally indicated that anti-δ can increase the humoral immune response to high doses of simultaneously injected antigen but does not induce polyclonal B cell activation. The recent availability of hybridoma produced anti-δ antibodies[20,21] and murine IgD secreting plasmacytomas[22] has made it possible for us to re-examine in vivo activation of murine lymphocytes by anti-δ antibodies using anti-δ concentrations similar to those achieved in in vitro activation experiments. We find that within 24 hrs after i.v. injection of 800 μg of affinity purified rat anti-mouse δ (DEM8) antibody splenic B cells have increased their quantitites of cell surface (s) Ia antigen, their size, and their rate of DNA synthesis. All three processes are T-independent. Six-seven days after anti-δ injection a second phase of activation is observed which is T-dependent. Splenic T cells as well as B cells are found to be proliferating, a 3-4 fold increase in the number of spleen cells is seen, large numbers of B cells with sIgG are found, and a polyclonal increase in the secretion of both

IgM and IgG is observed. This state of polyclonal immune activation persists as long as anti-δ antibody remains in circulation; once injected anti-δ is catabolized the immune system reverts to its initial level within one week.

GENERAL PROCEDURES

Following i.v. injection of 800 μg of GaMδ or control antibody BALB/c mice were sacrificed after varying periods of time. Portions of spleen and lymph nodes were formalin-fixed, sectioned, stained with hematoxylin and eosin and examined microscopically. Single cell suspensions prepared from these organs were analyzed for volume with a Coulter Channelizer and for DNA content by staining with an ethanolic solution of Mithramycin and determining cellular fluorescence intensity with a Becton-Dickinson FACS II fluorescence activated cell sorter. Cells were analyzed for surface markers by staining with an FITC-labeled monoclonal antibody specific for Thy 1.2 (Clone 30H-12) or with FITC-labeled affinity purified $F(ab')_2$ fragments of rabbit antibodies specific for mouse δ, μ, or γ chains or keyhole limpet hemocyanin (control) or with A.TH anti-A.TL (anti-Ia) or normal mouse serum (control) followed by FITC-labeled rabbit anti-mouse γ, after which the percentage of cells positive for each surface marker as well as the median fluorescence intensity of specifically stained cells was determined with a FACS II. Surface Ig was also studied by radioautographic analysis of an SDS-PAGE electropherogram of an immunoprecipitated NP-40 extract of ^{125}I surface labeled spleen and lymph node cells from anti-δ injected and control mice. The percentages of cells with intracytoplasmic IgM or IgG were determined by staining methanol fixed cytocentrifuge preparations of these cells with the appropriate FITC-labeled antibodies and examining the stained preparations by fluorescence microscopy. In some experiments the percentages of cells secreting IgM or IgG were determined by the Protein A reverse plaque assay and the percentages of cells secreting IgM anti-TNP were determined by a modified Jerne plaque assay. In some experiments mice were injected with ^{3}H-thymidine 18-24 hrs prior to sacrifice and the amount of ^{3}H incorporated per 2×10^{6} cells was analyzed by scintillation spectroscopy.

RESULTS

I. Early Events. Twenty-four hrs after injection of GaMδ the ratio of white pulp: red pulp in spleen is greatly increased and the small B lymphocytes of the mantle layers of spleen and lymph node follicles appeared to have differentiated into cells with increased amounts of pale staining cytoplasm

and large pale nuclei with prominent nucleoli. Surface IgD was almost totally modulated from splenic B lymphocytes, which retained most of their sIgM. No significant changes were seen in percentages of $sIg\delta^+$ or sIa^+ cells, although the fluorescence intensity of sIa staining was markedly increased (Table 1).

TABLE I

EFFECTS OF GaMδ ON SPLEEN CELL SURFACE MARKERS

Day*	Antibody Injected	Percent Positive** (Median Fluorescence Intensity) IgD	IgM	IgG	Ia	Thy 1
1	NI G IgG	55.3 (100)	55.8	3.8	51.1 (71)	N.D.
	GaMδ	<2	47.1	5.9	47.5 (125)	N.D.
3	NI G IgG	50.0 (75)	52.5	3.6	49.0 (83)	33.6
	GaMδ	9.3 (32)	64.3	2.8	63.5 (156)	21.8
7	NI G IgG	53.8 (117)	55.5	5.6	59.8 (60)	34.3
	GaMδ	9.9 (30)	43.4	38.1	†	27.8
14	NI G IgG	54.7 (96)	55.3	7.4	N.D.	30.6
	GaMδ	37.7 (73)	40.8	10.3	N.D.	45.7
18++	NI G IgG	47.5	51.5	5.8	N.D.	32.8
	GaMδ	2.3 (38)	20.0	24.8	†	30.3

* Days after injection of 800 µg of normal goat IgG or GaMδ.

** Pools of spleen cells from 3 mice were stained directly for IgD, IgM, IgG, or Thy 1, or indirectly for Ia (ATH anti-ATL + FITC-RaMγ) and analyzed with a fluorescence activated cell sorter. Median fluorescence intensity is a measure of quantity of marker present on cells expressing that marker. N.D. = Not done.

† Not analyzable because of the high percentage of cells staining directly for IgG.

++ Mice in this study were injected with 800 µg of normal goat IgG or GaMδ on days 0, 1, 3, 7, 11 and 14.

These changes were associated with marked increases in spleen cell size (Table 2) and DNA synthesis (Table 3). Twenty-four hours after GaMδ injection the percentage of spleen cells with greater than 2C DNA was more than 2 fold increased as compared to control values (Table 3). Three days after GaMδ injection spleen cells from anti-δ treated mice showed 4 times more *in vivo* ^{3}H-thymidine incorporation on a per cell basis than spleen cells from control

TABLE 2

EFFECT OF GaMδ ON SPLEEN CELL SIZE

Day*	Antibody Injected	Percent of Spleen Cells with a Volume of† 113-248 μ^3	253-423 μ^3	429-626 μ^3
1	Nl G IgG	74 ± 1	21 ± 1	6 ± 3
	GaMδ	51 ± 1	34 ± 1	15 ± 1
3	Nl G IgG	82 ± 2	15 ± 1	3 ± 0.2
	GaMδ	37 ± 3	44 ± 1	18 ± 2
7	Nl G IgG	85 ± 2	13 ± 1	2 ± 0.5
	GaMδ	29 ± 2	46 ± 1	24 ± 2
14	Nl G IgG	84 ± 1	14 ± 0.9	2 ± 0.3
	GaMδ	79 ± 4	18 ± 3	3 ± 0.6
13**	Nl G IgG	76 ± 7	20 ± 5	5 ± 2
	GaMδ	35 ± 3	43 ± 1	22 ± 4

*Days after injection of 800 µg of normal goat IgG or GaMδ.

**Mice were injected with 800 µg of normal goat IgG or GaMδ on days, 0,1,5,7,10,11 and 12.

†Arithmetic mean ± standard deviation of spleen cells from 3 mice.

mice. Sorting of these cells into sIa^+ and sIa^- fractions prior to Coulter analysis and scintillation spectroscopy demonstrated that only the sIa^+ cells from GaMδ treated mice showed increased size and ^{3}H-thymidine incorporation. These events appeared to be T-independent, since congenitally athymic (nu/nu) mice and mice tolerized to goat IgG by i.v. injection of 2 mg of ultracentrifuged goat IgG 2 weeks prior to GaMδ injection behaved similarly to normal non-tolerized GaMδ treated mice during the first 3 days after anti-δ injection. Anti-δ induced increases in sIa density, B cell size, and B cell DNA synthesis, are, to some extent, independent events in B cell activation. Injection of 100 µg of GaMδ induced a substantial increase in sIa density but not B cell size. B cells from mice with the CBA/N X-linked immune defect[23] increased in size in response to GaMδ both *in vivo* and *in vitro* (A. DeFranco and W.E. Paul, personal communication) but did not show increased DNA synthesis.

II. Late Events. Six - seven days after GaMδ injection the immune system demonstrated further evidence of polyclonal activation. The frequency of $sIgG^+$

TABLE 3

EFFECTS OF GaMδ ON SPLEEN CELL NUMBER AND DNA SYNTHESIS

Day*	Antibody Injected	Cell Number x 10^{-6}		^{3}H-Thy CPM/2 x 10^6 Cells†		Percent >2C DNA**	
1	Nl G IgG	101	(1.13)***	787	(1.10)***	4.7	(1.60)***
	GaMδ	86	(1.10)	714	(1.30)	10.4	(1.26)
3	Nl G IgG	78.6	(1.06)	548	(1.17)	5.5	(1.23)
	GaMδ	119	(1.10)	2,257	(1.18)	12.2	(1.08)
7	Nl G IgG	75.9	(1.17)	551	(1.17)	5.5	(1.11)
	GaMδ	352	(1.22)	1,866	(1.85)	12.1	(1.34)
14	Nl G IgG	111	(1.08)	N.D.		3.4	(1.08)
	GaMδ	115	(1.15)	N.D.		4.6	(1.17)
13††	Nl G IgG	169	(1.53)	N.D.		6.4	(1.70)
	GaMδ	263	(1.63)	N.D.		22.6	(1.21)

*Days after injection of 800 µg of normal goat IgG or GaMδ.
**Determined by fluorescence intensity after staining with mithramycin. Geometric mean and standard deviation of spleen cells from 3 mice.
†CPM of ^{3}H-Thymidine/2 x 10^6 Nucleated Spleen Cells 18 hrs after i.v. injection of 100 µCi of ^{3}H-Thymidine.
††Mice were injected with 800 µg of normal goat IgG or GaMδ on days 0,1, 5,7,10,11, and 12.
N.D. = Not Done.

spleen cells increased to 4-8 times the normal level (Table 1). Some of this increase may have been due to formation of GaMδ - mouse anti-goat Ig immune complexes that bound to Fc receptors. However, the appearance of a large percentage of Thy 1^- $sIgM^-$ cells at this time favors the possibility that increased numbers of B cells with intrinsic sIgG were appearing. In addition, SDS-PAGE analysis of reduced ^{125}I-labeled sIg from these cells indicated the presence of a major band with mobility slightly faster than that of δ chain and slower than that of the heavy chain of serum IgG_{2a}. This major band, which is found only in trace amounts on lymphoid cells from control mice, has a mobility similar to that of previously described surface γ_{2a} from murine tumor cell lines.[24,25]

Spleen cells from GaMδ treated mice 7 days after injection continued to show increased size and DNA synthesis. However, analysis of FACS purified

TABLE 4

EFFECT OF GaMδ ON GENERATION OF SPLEEN CELLS THAT EXPRESS CYTOPLASMIC IG

Day*	Antibody Injected	Percent of Cells Expressing Cytoplasmic IgM	IgG
5	Nl G IgG	0.9 (1.45)†	<0.2
	GaMδ	2.2 (1.53)	0.4 (2.03)
7	Nl G IgG	0.4 (1.09)	0.5 (2.14)
	GaMδ	2.5 (1.67)	15.7 (1.28)
14	Nl G IgG	0.2 (1.46)	0.3 (1.88)
	GaMδ	0.7 (1.29)	1.4 (1.60)
13**	Nl G IgG	1.5 (1.14)	3.1 (1.54)
	GaMδ	6.9 (1.39)	33.0 (1.26)

*,**Days after injection of 800 µg of normal goat IgG or GaMδ. Mice were injected with 800 µg of normal goat IgG or GaMδ on days 0,1,5,7,10,11, and 12.

†Geometric mean and standard deviation of spleen cells from 3 mice.

sIa^+, sIa^-, $sThy\ 1^+$ and $sThy\ 1^-$ spleen cell populations from these mice showed that both B and T cells had now increased in size and rate of DNA synthesis. These findings are consistent with the observation that absolute numbers of both B and T cells in spleen were now substantially increased (calculated from Tables 1 and 3).

Increases in synthesis of IgG and IgM by spleen cells were also noted 7 days after GaMδ injection. This was evident from the percentages of cells that contained intracytoplasmic IgM and IgG (Table 4) as well as from approximately 10 fold increases in frequencies of Ig secreting cells (determined by the protein A reverse plaque technique) total Ig secretion (determined by in vitro incorporation of ^{3}H-leucine into IgM and IgG), and cells secreting IgM anti-TNP antibodies. This last finding is of particular importance as it indicates that the increased Ig synthesis stimulated by GaMδ represents a polyclonal antibody response rather than simply an enhanced anti-goat Ig response. In congenitally athymic mice no increase in the frequency of $sIgG^+$ lymphocytes, rate of lymphocyte DNA synthesis, spleen cell number, or Ig synthesis were observed 7 days after GaMδ injection. Similarly, mice "tolerized" with 2 mg of ultracentri-

fused goat IgG showed much less evidence of polyclonal activation 7 days after GaMδ injection than did nontolerized mice.

III. Reversal of Activation. Ten days after GaMδ injection circulating anti-δ was no longer detectable and considerable numbers of $sIgD^+$ cells were seen. Decreases in the size of splenic lymphocytes and a reduction in the number and frequency of $sIgG^+$ cells were also observed. By 14 days after anti-δ injection these parameters (Table 1-3), as well as the frequency of cells with intracytoplasmic Ig (Table 4) and the histologic appearance of the spleen had almost completely normalized. One abnormality was present, however; the percentages of B lymphocytes in spleen and lymph nodes were considerably below the unstimulated values.

If multiple injections of GaMδ were given to maintain circulating levels of this antibody the activated immune state persisted. As many as 40% of spleen cells contained intracytoplasmic Ig 13 days after the initial anti-δ injection and histologic sections of spleen demonstrated many lymphoid cells with relatively large amounts of darkly staining cytoplasm in both the red and white pulp.

DISCUSSION

Our data suggest that in vivo B cell activation by soluble anti-δ proceeds through 2 phases. The first, which includes increases in sIa density, cell size, and rate of DNA synthesis appears to be T-independent. The second phase, which includes a further increase in rate of DNA synthesis, acquisition of sIgG, and Ig secretion, appears to be T-dependent and, to a large extent, carrier dependent, which suggests that T cell help specific for goat Ig may be polyclonally focused onto B cells by GaMδ antibody.

Our results have a number of implications. First, they establish the validity of a two stage model of B cell activation[26,27] under physiological conditions. Second, they support the view that B cells acquire sIgG in the process of differentiating into IgG secreting cells.[28] Third, they indicate that in the presence of endogenously generated T cell help, the interaction of a ligand with sIgD can activate B lymphocytes to secrete antibody even when that ligand does not interact with sIgM. As other papers in this volume suggest that the interaction of ligand with sIgD is not required for B cell differentiation into antibody secreting cells[29,30] it seems likely that the interaction of ligand with either sIgD or sIgM can lead to B cell terminal differentiation in the presence of appropriate helper factors. This interpretation suggests

that blocking of in vitro immune responses by anti-μ or anti-δ antibodies is due to a direct suppressive effect induced by the binding of these ligands to sIgM or sIgD rather than to their blocking of antigen binding by sIgM or sIgD. In previous papers we have presented our belief that an important functional difference between sIgD and sIgM is the relative strength of the suppressive effect generated by ligand - sIg interaction, with the binding of ligand to sIgM producing the greater suppressive effect.[19]

It is of interest that the anti-δ concentrations required to achieve polyclonal activation in vivo and in vitro are higher than the concentration of some antigens that are required to induce specific antibody secretion under similar conditions. The low epitope density and helper factor stimulating abilities of soluble anti-Ig antibodies relative to some antigens may contribute to this difference. For example, Parker has found that binding anti-Ig antibodies to an insoluble matrix and adding helper factors to an in vitro culture system substantially reduce the quantities of anti-Ig antibody required for B cell activation.[14]

The mechanisms by which T lymphocytes are stimulated to proliferate in the second phase of anti-δ activation are not clear. Possibilities include production of factors by activated B cells that induce T cell proliferation, induction of T cell proliferation by goat Ig-mouse anti-goat Ig complexes, and stimulation of an autologous MLR by new antigenic determinants on GaMδ modified B lymphocytes.

The reasons for the collapse of the activated immune system once anti-δ is established are also obscure. Histologic data suggest that cells die in situ in the spleen, rather than migrate to other organs. Specific or non-specific suppressor macrophages or T lymphocytes, killer cells specific for activated lymphocytes, and an immunologic network in which polyclonal activation results in secretion of Ig molecules that induce "complimentary" suppression by binding to each others idiotypic determinants may all contribute to the collapse. None of these mechanisms, however, leads inevitably to suppression of the activated immune state, since that state appears to be maintained as long as circulating levels of GaMδ are present.

In many ways polyclonal activation by GaMδ antibody seems to closely parallel activation of antigen binding clones by a T-dependent antigen. The relative ease with which the in vivo GaMδ system provides large populations of activated B and T lymphocytes should therefore be of great value in the study of antigen activated cells and the molecular bases of B lymphocyte activation and regulation.

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